FURTHER CHEMICAL ASPECTS OF THE SENSITIZATION AND ACTIVATION REACTIONS OF NEREIS EGGS

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The peculiar effects of 2,4,6-trinitrophenol (picric acid) in relation to the artificial activation of the eggs of *Nereis limbata* have been described in an earlier report (LeFevre, 1945). Discovery of these effects grew from a reinvestigation of a few experiments by Heilbrunn (1925) concerning the enhancing effect of acidification on the heat-activation of these eggs. Among a series of rather unrelated organic acids tested, only picric acid exerted a reliable activity of this sort, and further experimentation with this compound revealed a rather paradoxical set of properties with regard to the egg activation. Thus, at a concentration of about M/1000 in sea water, trinitrophenol prevented breakdown of the germinal vesicle, and accompanying cytoplasmic reorganization, ordinarily produced in these cells by exposure to heat (Just, 1915), excess potassium ion, or decalcifying agents (Wilbur, 1941). At the same time, exposure of the eggs to the same concentration of trinitrophenol rendered them subsequently hypersensitive to these same activating agents, so that upon removal from the acid they could be activated by exposure to these agents in doses too small to activate untreated eggs. This development of hypersensitivity was progressive with continued exposure to trinitrophenol, until after several hours the eggs were activated simply by removal to sea water, without application of any additional chemical or physical stimulant. The eggs remained in this state of hypersensitivity in the trinitrophenol for as long as three days, by which time in sea water they would long since have cytolyzed.

Various hypotheses were considered in an effort to integrate these diverse influences of trinitrophenol on the reactions of activation into a simple coherent pattern, in relation to some of the established factors relating to this process in the *Nereis* egg. The importance of the calcium ion in this connection (Heilbrunn and Wilbur, 1937; Wilbur, 1939, 1941) directed attention to the question of a calcium picrate complex formation, but there seemed to be no physicochemical basis for this; complications confronting interpretation of the effects of trinitrophenol in terms of a calcium-release theory are considered in the previous report (LeFevre, 1945). The least involved interpretation capable of explaining the behavior of the trinitrophenol seemed to be that this substance might form a reversible combination with some substance produced in the course of the cell's metabolic activities; that this substance could precipitate the breakdown of the germinal vesicle, but that it is normally removed by chemical reaction or by diffusion from the cell before activating concentrations are attained. This hypothesis would attribute the stimulating effects of the various agents to an increased rate of production of the activating metabolite, which can then take part in the critical reactions. Inhibition of this stimulation by trinitrophenol was attributed to formation of an inactive complex between the acid and the hypothetical activating substance. This interpretation accounted for the pro-

gressive development of hypersensitivity to activating agents during exposure to trinitrophenol, since removal from the acid bath to ordinary sea water, with accompanying rapid loss of picrate, would then release the accumulated activator, and would be equivalent to the application of an activating agent. The original report may be consulted for a more complete consideration of this hypothesis in the light of the earlier experiments. The present report is an extension of the above investigation in an attempt to elucidate the nature of the hypothetical reactions suggested by the original work.

Materials and Methods

Two main methods of approach have been employed: chemical disturbance of the egg-suspension medium, either during the sensitizing procedure with trinitrophenol or during application of chemical activating agents; and treatment of the eggs with substituted phenols other than picric acid, to ascertain the molecular specificity of the activities described for the latter.

The materials and general handling procedures have been previously described (LeFevre, 1945). In those experiments reported below involving treatment of egg-suspensions with gas mixtures, the appropriate quantities of carbon dioxide, unpurified commercial nitrogen, and air were drawn into a glass vessel of several liters capacity, by removal of water through a siphon; from this vessel the mixture was similarly forced in fine bubbles through 25–35 ml. of the egg-suspension in a 10–15 cm. column in an ordinary test tube. In addition to gas mixtures, specific metabolic inhibitors tested included potassium cyanide, sodium azide, sodium iodoacetate, hydroxylamine, *p*-chloromercuribenzoic acid, cupric chloride, urethane, and diethyl ether. Substituted phenols tested included 2,4,6-trinitrophenol, 2,4-dinitrophenol, *o*-nitrophenol, *p*-nitrophenol, 2,6-dichlor,4-nitrophenol, *o*-chlorophenol, and *p*-chlorophenol.

The artificial sea water medium used in some of the experiments was made up by mixing of several pure salt solutions isotonic with sea water: NaCl, 0.52 M, 500 volumes; KCl, 0.53 M, 10 volumes; $MgCl_2$, 0.37 M, 40 volumes; $MgSO_4$, 0.96 M, 15 volumes; $NaHCO_3$, 0.52 M, 2 volumes; $CaCl_2$, 0.34 M, 15 volumes. For those experiments involving upset of the normal proportions of the ingredients of sea water, corresponding changes were made in the relative volumes of the isotonic solutions mixed, so that the resultant surplus or deficit in total electrolyte content was taken up by all the other ingredients in their usual proportions.

Results

I. Experiments concerning reactions accompanying activation

a. Effects of anoxia

The dependence of the germinal vesicle breakdown response on aerobic processes was tested only in the case of activation by removal to sea water from prolonged exposures to trinitrophenol (10^{-3} M in sea water). There was no evidence of activation when the eggs were transferred to sea water through which a mixture of 95 per cent N_2, 5 per cent CO_2 had been passed for about thirty minutes, while 100 per cent activation was ordinarily obtained in undisturbed sea water or in sea water

gassed with air or 95 per cent air, 5 per cent CO_2. This inhibition of activation in a nitrogen atmosphere was reversible for several minutes; Table I shows a typical instance of this, in which the eggs, upon removal from the anoxic bath to ordinary sea water, were activated in progressively diminishing numbers as the interval in the anoxic medium was extended.

b. Effects of metabolic inhibitors

The usual chain of events following removal of eggs from trinitrophenol baths to sea water was similarly inhibited by ether, potassium cyanide, or sodium azide; these inhibitors, however, did not prevent a slight elevation of the membrane, and the very first indications of the onset of nuclear reorganization, as previously described in eggs exposed to mixtures of sea water and isotonic KCl or sodium citrate in the presence of trinitrophenol (LeFevre, 1945). This initial disturbance was "frozen" at a very early stage, so that the germinal vesicles retained their identity, and none of the cytoplasmic rearrangements consequent to vesicular breakdown occurred. If, subsequently, the inhibitor was removed, even after several hours of

TABLE I

Reversible inhibition of activation by anoxia

Sea water under atmosphere of:	Per cent activation
Air	98
95% N_2, 5% CO_2	0
Removed, exposed to air	
after 1 min.	76
3 min.	60
5 min.	54
10 min.	32

Activating procedure: eggs removed to sea water, under atmosphere indicated, after exposure for three hours to trinitrophenol, M/1000, in sea water.

such suspended activation, the usual processes continued normally; i.e., the inhibition was entirely reversible. In the case of ether, in fact, gradual evaporation of the narcotic led ultimately to a resurgence of the activation reactions, without actual removal of the eggs to fresh sea water. A typical set of experiments is presented in Table II. It may be noted that this reversibility outlasted that observed in anoxia by a wide margin, but this may have been attributable to the more rapid onset of irreversible injury to the eggs in the absence of oxygen. The critical concentrations for inhibition were those used in the experiments cited in Table II.

The same concentrations of ether, azide, or cyanide were equally effective in preventing activation in a few tests made with mixtures of sea water and isotonic KCl or sodium citrate (Table II); reversibility of this inhibition was not tested, but the high percentages of "incipient activation" observed would indicate the same state of affairs as seen with these inhibitors following the sensitization procedure. Hydroxylamine, at 10^{-2} M, also prevented activation by these agents, but the inhibition was irreversible, and although there was no immediate obvious morphological change, inhibition in this instance probably indicated nothing more than complete inactivation of the cells.

The other inhibitors tested in this same connection, rather than preventing activation, proved to be themselves activating agents. The same nuclear and cytoplasmic changes accompanying treatment with excess potassium, heat, etc., were seen when the eggs were exposed to sodium iodoacetate, at ca. $2 \cdot 10^{-2}$ M, or to urethane, at ca. $5 \cdot 10^{-3}$ M. At these concentrations, and throughout a fairly extensive range above these figures, 90–100 per cent germinal vesicle breakdown was regularly observed and was frequently followed by extensive irregular cleavage, though no swimming forms were found. Activation by these means resembled that induced by other agents in that it failed in the presence of trinitrophenol at 10^{-3} M. *p*-Chloromercuribenzoate or $CuCl_2$ (like iodoacetate, inhibitors of sulfhydryl activity) also activated the eggs, at concentrations in the neighborhod of 10^{-5} M; but this reaction was less easily reproducible than with the other stimulating "inhibitors," as the threshold concentration for activation was only slightly lower than the lytic

TABLE II

Reversible inhibition of activation by metabolic inhibitors

Activating agent	Inhibitor	Per cent activation		
		Without inhibitor	With inhibitor	Inhibitor removed after 7 hours
Sea water, after 5 hours in trinitrophenol, M/1000, in sea water	Ether, 1%	100	0, 80*	100
	KCN, 10^{-3} M	100	0, 100	100
	NaN_3, 10^{-2} M	100	0, 50	100
KCl, 20 vol. isotonic, to 80 vol. sea water	Ether, 1%	100	0, 70	
	KCN, 10^{-3} M	100	0, 75	
	NaN_3, 10^{-2} M	100	0, 100	
Sodium citrate, 25 vol. isotonic, to 75 vol. sea water	NaN_3, 10^{-2} M	94	0, 90	

* The second figure in this column is the approximate percentage of eggs in the inhibitor showing the condition of "incipient activation" (elsewhere described).

concentrations, and there was some variability in the effective concentrations for different batches of eggs. A further difference in the activation in these instances is that that induced by Cu^{++} or *p*-chloromercuribenzoate was not interfered with by trinitrophenol. Thus activation by these agents appears not to be comparable to that otherwise induced, but more analogous to the prelytic activation seen in other instances (Loeb, 1913; Lillie, 1926).

c. Effects of ionic components of sea water

The regular response of the eggs upon removal to sea water after several hours' exposure to trinitrophenol, 10^{-3} M in sea water, raised the question of what components of sea water were essential to this response. If the eggs were removed from the conditioning bath not to sea water, but to isotonic solutions of either NaCl, KCl, $CaCl_2$, or $MgCl_2$, no stimulatory reaction was ever observed. Mixtures of

various proportions of these basic ingredients were then tested. In the absence of magnesium, there was always such a distortion of the egg contours, with extreme membrane elevation and discoloring, that it was difficult to analyze other differences that might appear. It was however evident that the addition of calcium, in the absence of magnesium, greatly augmented these disruptive changes, leading to decided cellular deformation and nuclear disarrangement resembling in some respects the changes that occur in activation, and frequently to extensive cleavage, but without any change in the appearance of the cytoplasm. The deleterious effects were further exaggerated in the presence of bicarbonate buffer, with a characteristic red-brown discoloration and vesiculation of the protoplasm. In an isotonic solution containing $MgCl_2$, NaCl, and $NaHCO_3$, with the Na^+, Mg^{++}, and HCO_3^- in proportions similar to those found in sea water, the eggs remained perfectly normal in appearance. But this mixture was not capable of inducing the reactions of activation in eggs transferred from a conditioning bath with trinitrophenol. Activation required the presence of at least a reasonable trace of calcium ion; and at a given cal-

TABLE III

Action of calcium and potassium ions in initiation of activation

Concentration of potassium ion	Per cent activation with calcium ion concentration of:			
	0*	0.01 M	0.02 M	0.04 M
0*	0	0	50	66
0.005 M	0	93	92	98
0.01 M	0	100	100	100
0.02 M	3	100	100	100

* On basis of absolute freedom of reagents from Ca^{++}, K^+-contamination, and ignoring small amount carried over in sea water with eggs. Medium was made up of NaCl, 0.47 M; $MgCl_2$, 0.037 M; $NaHCO_3$, 0.0026 M; plus $CaCl_2$, KCl as indicated for each case. Eggs removed to medium indicated after exposure for 7 hours to trinitrophenol, M/1000, in sea water.

cium ion level the reaction was augmented by increased potassium ion concentration, as in the typical experiment shown in Table III. If both calcium and potassium were present at M/100, the reaction was as easily elicited as in sea water. To some extent, increased concentration of magnesium ion antagonized this stimulatory activity, so that more calcium and potassium were required to elicit the same percentage of response. The specific necessity for calcium was verified by varying its concentration in the otherwise fairly complete artificial sea water medium described in an earlier section; reduction of the calcium level to half the normal figure produced a noticeable diminution in the percentage of response, although there was still some degree of response even with only one-tenth of the normal calcium concentration.

The necessity of calcium, in initiating the stimulatory changes upon removal from exposure to trinitrophenol, was immediate, as shown in Table IV. Addition of calcium to the system only 60 seconds after the removal of the trinitrophenol did not result in a significant amount of activation. Thus in this sense the inhibition of activation by calcium-lack may be said to be irreversible. This effect is not, however, attributable to general injury to the cells because of the lack of calcium, as the

same cells may subsequently be activated by any of the usual procedures. Also, the eggs remained fertilizable for as long as eight hours in artificial sea water as nearly Ca-free as the purity of the reagents permitted. Such eggs, however, always lost their fertilizability and cytolyzed some time before those in the control medium.[1]

Table IV

Immediate necessity of calcium in activation following sensitization

Condition	Per cent activation
In artificial sea water	50
In Ca-free artificial sea water	0
Removed, to complete artificial sea water, after 15 sec.	51
30 sec.	36
60 sec.	10
90 sec.	0
120 sec.	0
180 sec.	0

Eggs removed to medium indicated after exposure for 24 hours to trinitrophenol, M/1000, in sea water.

In a similar manner, depletion of the calcium content of the medium prevented activation of the eggs by Wilbur's methods, addition of isotonic KCl or sodium citrate to the sea water medium. Citrate activation was especially sensitive to low calcium concentration, failing entirely if as little as ⅔ of the calcium was removed; this was particularly curious, since the citrate in itself would be expected to render unavailable most of the calcium in the medium.

II. *Experiments concerning reactions accompanying sensitization*

a. *Effects of anoxia*

Comparisons were made of the rates of development of sensitivity to sea water, during exposure to trinitrophenol, in the presence of various gas mixtures. Gassing the trinitrophenol solution (10^{-3} M in sea water) with nitrogen, or with 95 per cent nitrogen, 5 per cent carbon dioxide, led to a decidedly earlier development of sensitivity (Table V); but with more prolonged exposures, the eggs in the anoxic baths became irreversibly inactivated, and began to cytolyze, long before any evidence of damage appeared in the control aerated dishes. Increased percentage of CO_2 in mixtures with air, at least up to 50 per cent CO_2, similarly enhanced the development of sensitization, and did not appear to injure the eggs subsequently. It was frequently observed in other experiments that the packing of eggs in considerable thicknesses at the bottom of the container, during exposures to trinitrophenol, led to more rapid development of sensitivity than appeared when more dispersed, thinner layers of eggs were used. It seems likely that this effect is to be attributed

[1] On the other hand, less pronounced depletion of the calcium, down to about 1/100 the normal level, progressively delayed the onset of cytolysis; this effect may be due simply to the calcium requirements of micro-organisms responsible for the disintegration of the eggs, but Schechter (1937a, b) has described similar effects of Ca^{++}-reduction in a number of species of commonly used marine eggs.

TABLE V

Enhancement by anoxia of sensitization in trinitrophenol

Duration of sensitizing bath min.	Per cent activation following sensitization under atmosphere of: 95% N_2 5% CO_2	95% air 5% CO_2
4	0	0
16	7	1
41	100	6
59	100	63

Eggs removed to fresh sea water after indicated exposure to trinitrophenol (10^{-3} M in sea water) under atmosphere described.

to the higher CO_2 or the lower O_2 tension (or both) in the immediate environment of the cells packed in greater density.

b. *Effects of metabolic inhibitors*

Short of blocking subsequent activity by killing the cells (as indicated by loss of fertilizability, rapidly followed in most instances by disintegration), no reliable influence on the rate of sensitization in trinitrophenol was observed in the presence of ether, urethane, or sodium iodoacetate. (See section *d* below.) Hydroxylamine, at 10^{-2} M, or potassium cyanide, at ca. 10^{-3} M,[2] markedly enhanced the rate of sensitization, the more so as the concentration was increased; a less reliable similar activity was seen with sodium azide, in the neighborhood of $5 \cdot 10^{-3}$ M. Table VI shows a typical set of results with various concentrations of KCN. In all of these cases, the stimulatory action was superseded by lethal effects, more readily at the higher concentrations, the maximum stimulating action then passing progressively to the lower concentrations, as in the instance presented in Table VI. This cytolysis occurred following the transfer to sea water, not in the trinitrophenol baths containing the inhibitor; this is as would be expected if a great excess of the activator led to cytolysis rather than activation, as was also suggested in the older experiments.

TABLE VI

Enhancement by cyanide of sensitization in trinitrophenol

Duration of sensitizing bath min.	Per cent activation when sensitized with KCN at concentration of:			
	$5 \cdot 10^{-3}$ M	10^{-3} M	$2 \cdot 10^{-4}$ M.	0
72	100	27	39	4
127	52	68	61	2
432	0	97	75	62
1554	Cytolyzed	Cytolyzed	100	99

Eggs removed to fresh sea water after indicated exposure to trinitrophenol (10^{-3} M in sea water) with added KCN as shown.

[2] Cyanide and trinitrophenol reacted slowly in these preparations, progressively developing an amber color of unknown significance, over a period of several hours. With higher concentrations of cyanide, the rate of development of this discoloration was correspondingly higher.

c. Effects of ionic components of sea water

Following the experiments described above, in which it appeared that the calcium ion was the specifically essential component of sea water in permitting the initiation of activation, possible involvement of this ion in the reactions of sensitization was tested. Removal of calcum ion from the artificial sea water medium, containing trinitrophenol at M/1000, produced no evident change in the rate of sensitization; this absence of effect was noted with calcium contents as low as 1/60 the normal level. More complete removal was impracticable, since in such low-Ca^{++} media the trinitrophenol became rapidly toxic to the cells and led to cytolysis before the sensitization had gotten well under way. This protective effect of calcium against destruction of the eggs by the acid was evident in comparison of the normal medium with that containing as much as 1/10 the normal amount of calcium ion.

Since it was evident from experiments considered above that increased CO_2 tension hastened sensitization in trinitrophenol, it seemed expedient to test the effects of alterations in the related buffer system. But simple neutralization of the trinitrophenol (readjusting the pH from 6.7–7.0 back to 8.0 as in sea water) had no demonstrable effect on the rate of sensitization. In nearly all of the experiments with trinitrophenol, this neutralization was routinely performed. In a single experiment, the amount of bicarbonate in equilibrium with atmospheric CO_2 was varied in artificial sea water media, from zero to three times the normal amount, and eggs were exposed to neutralized trinitrophenol, 10^{-3} M, in each of these mixtures. Throughout this range, the sensitization rate was more rapid, the more bicarbonate present (Table VII). The effect of CO_2 is thus probably not to be attributed to its acidifying effects in the medium, but to its action after passing intracellularly, as in the effects described by Jacobs (1920).

TABLE VII

Effect of bicarbonate on sensitization in trinitrophenol

Duration of sensitizing bath min.	Per cent activation following sensitization in trinitrophenol, 10^{-3} M, in artificial sea water with bicarbonate content of:			
	3 × usual	Usual (0.0018 M)	0.3 × usual	None
25	0	0	0	0
92	0	0	0	0
156	2	0	0	0
212	38	4	3	1
405	100	77	3	0
1307	100	100	100	100

d. Effects of stimulating agents

One deduction from the hypothesis of the activator-substance (LeFevre, 1945), subject to experimental test, is that the activator should accumulate more rapidly if the eggs in the trinitrophenol bath are simultaneously exposed to an agent which would bring about activation in the absence of the inhibitor. As previously re-

ported, this proved to be the case in only about 1/5 of the tests when heat was used as the activating agent, the remaining 4/5 of the results showing no significant differences in either direction.

The same procedure applied to mixtures of sea water and isotonic KCl or sodium citrate gave different results with the two agents. With addition of extra potassium to the sensitizing picrate baths, there was invariably a more rapid development of the capacity to react upon removal to sea water, similar to that shown with other agents in Tables V, VI, and VII. This was in accord with the predictions of the hypothesis; in no case, however, did such a difference appear in the presence of the citrate stimulant. Also, as noted above, the stimulants urethane and iodoacetate did not regularly enhance the development of sensitivity in trinitrophenol; each of these substances exerted this effect in a few instances, and in no case produced a slowing of the sensitization, so that their position in this regard is the same as that of the heat stimulus originally investigated.

III. Experiments concerning the effectiveness of substituted phenols other than picric acid

a. Blocking of activation

Several substituted phenols differing from trinitrophenol in the nature or position of the substituted groups on the phenol were compared, all at 10^{-3} M, the concentration at which standard experimentation with trinitrophenol was carried out.[3] 2,4-Dinitrophenol and *p*-nitrophenol were more effective than trinitrophenol in blocking activation by either KCl-sea water mixtures or sodium citrate-sea water mixtures; in one case only, *p*-nitrophenol allowed a small number of eggs to reach the stage previously designated as "incipient activation." *o*-Nitrophenol was almost as effective against citrate activation, and was superior in this respect to trinitrophenol; in spite of this fact, *o*-nitrophenol had absolutely no influence on activation by excess potassium. 2,6-Dichlor,4-nitrophenol was approximately equivalent to 2,4,6-trinitrophenol in its inhibitory action, usually allowing a moderate percentage of "incipients." *o*-Chlorophenol and *p*-chlorophenol usually prevented activation, but these effects appeared to be attributable to irreversible damage to the eggs. Prolonged exposure to the chlorophenols led to cytolysis, the initial stages of which, as noted by many investigators in artificial parthenogenesis, resemble in some respects the early stages of activation, so that it at times appeared upon cursory examination that these chlorophenols augmented the stimulating action of the chemical agents used. Phenol itself had no inhibitory effect whatsoever at this concentration.

In summary, the inhibitory effect of picric acid was duplicated by each tested phenol which had a nitro-group in the para-position; the only comparable action

[3] If neutralized, trinitrophenol at concentrations as high as 10^{-2} M were tolerated for many hours, and were very effective in blocking and sensitizing, but the concentration of 10^{-3} M gave the most prolonged effects without cell damage. Increasing the concentration past 10^{-3} M did not increase the rate of development of sensitivity to sea water in eggs exposed to the trinitrophenol; this independence of the rate of sensitization from the trinitrophenol concentration is in harmony with the hypothesis advanced in the discussion concerning the mode of action of the trinitrophenol.

seen in the absence of this group was that of *o*-nitrophenol with respect to citrate activation.

b. Sensitization to sea water

At 10^{-3} M, *o*-nitrophenol, 2,4-dinitrophenol, *o*-chlorophenol, or *p*-chlorophenol had no sensitizing effect on the eggs in exposures up to 24 hours or until the onset of cytolysis. Cytolysis occurred within a few hours in *p*-chlorophenol, after about 20 hours in *o*-chlorophenol or dinitrophenol, but no sooner in *o*-nitrophenol than in sea water. *p*-Nitrophenol showed some sensitizing activity, as indicated in

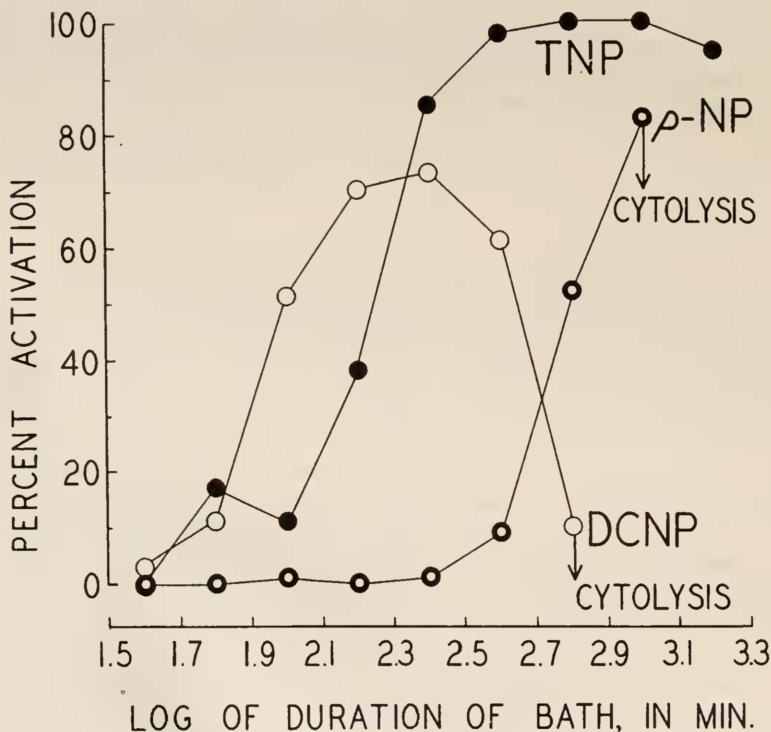

FIGURE 1. Comparison of sensitization rates in the three effective substituted phenols found.

Eggs removed to sea water following bath in either 2,4,6-trinitrophenol (TNP), *p*-nitrophenol (*p*-NP), or 2,6-dichlor,4-nitrophenol (DCNP); all at 10^{-3} M, in sea water.

Each point is the average of all experiments (usually three or four) performed in the logarithmic time interval marked at the base-line. Each individual experiment compares the action of the three phenols on the same batch of eggs.

Figure 1, but only after about 10 hours, and this effect disappeared rather rapidly, so that cytolysis began to be evident at about 20 hours. The dichloronitrophenol sensitized the eggs to sea water as rapidly as, or perhaps a little more rapidly than, trinitrophenol, but, like *p*-nitrophenol, led to cytolysis within about 20 hours (Fig. 1).

Thus, it seemed apparent that again the $-NO_2$ group in the para-position on the phenol was the primary essential, the groups in the ortho-positions exerting only modifying influences on the sensitizing reactions. However, unlike the situation observed in the study of inhibition of activation, there was no sensitizing activity evident with 2,4-dinitrophenol, which contains the apparently critical group. This is especially odd in view of the fact that either the addition of another $-NO_2$ in the 6-position, or removal of the $-NO_2$ from the 2-position, produces a highly active substance (many swimming "larvae" were obtained from the use of trinitrophenol or *p*-nitrophenol). Also, substitution of $-Cl$ for both of the $-NO_2$ groups in the ortho-positions did not interfere wth the sensitization activity.

However, of all these, only 2,4,6-trinitrophenol (picric acid) was effective in the lengthy preservation of the eggs against death and cytolysis; the others were all, in fact, somewhat toxic, leading to cytolysis within the first day of exposure. This may mean that the two nitro-groups in the ortho-positions serve to detoxify the molecule, perhaps preventing other reactions not basically related to those involved in the reversible inhibition and sensitization under study.

Discussion

None of the newly acquired data is antagonistic to the hypothesis developed from the results previously reported, involving the concept of the metabolite activator-principle. However, no crucial experimental test of this hypothesis has as yet been conceived; the present data concern the general nature of the reactions accompanying chemical activation and sensitization of the eggs, insofar as this is revealed by aberrations in these reactions with changes in the chemical environment.

Thus it is apparent that some phase of the reactions accompanying the process of germinal vesicle breakdown involves an oxidation employing molecular oxygen, probably through a cytochrome system, since this process was reversibly prevented by anoxia, cyanide, or azide. However, as noted by Barron (1932), in the activation of *Nereis* eggs by actual fertilization by sperm in very complete anoxia, this inhibition affects not the initiation of activation, but the later nuclear changes and consequent development. Diethyl ether also prevented these reactions, but there is less specificity in its action, so that interpretation of this inhibition is more indefinite.

With the exception of ether, the same inhibitors which prevented activation also hastened the onset of sensitization of the eggs exposed to trinitrophenol, so that the cells were activated, upon removal from the acid to plain sea water, following a shorter stay than required in the absence of the inhibitors. This fact is readily incorporated into the general hypothesis, since inhibition of the activating reactions would be expected to lead to more rapid accumulation of the activator-trinitrophenol complex, by eliminating one of the routes by which the free activator might be otherwise removed. Interpretation might also be sought on the basis of the acidifying effects of these inhibitors intracellularly, with consequent release of Ca^{++} from combination with cellular proteins. The fact that the inhibitors never appeared to induce sensitization in themselves, together with the temporal characteristics of the sensitization, would necessitate a rather unwieldy complex of assumptions about the behavior of the Ca^{++} and trinitrophenol, in application of these interpretations (LeFevre, 1945).

The observed results would however be expected if there were any alternative pathway by which the activator could be removed, either by chemical reaction or by diffusion from the cell; or simply if the reaction in which the activator is released were reversible and governed by mass action. In the latter case, accumulation of the activator in the presence of these metabolic poisons would be self-limited, whereas large amounts could be accumulated in inactive form with trinitrophenol, and this process would be accelerated by addition of the inhibitors; this would account for all the relevant experimental results with a minimum of independent hypotheses.

As noted above, the metabolic inhibitors which prevented activation exert their influence not on the immediate activating disturbance (visible at the egg surface) but on the immediately subsequent cellular reorganization. On the other hand, inhibition of the same over-all process ("activation") by removal of calcium ions from the medium acted at the very earliest stages in the chain of events. The absence of calcium ion at the critical instant of potential initiation of activation was thus an irreversible disturbance, and the cell did not react upon replacement of the missing element unless a second stimulus was applied. Heilbrunn (1925), Heilbrunn and Wilbur (1937), and Wilbur (1939, 1941) concluded from experiments along various lines that the reaction in question is dependent on rearrangement of the intracellular calcium with respect to the protoplasmic colloids, with accompanying changes in viscosity. The modifying influences of potassium and magnesium ions, as reported above, are in keeping with the general pattern of these cations in affecting colloidal reactions with calcium ion, as described by Heilbrunn in numerous cellular reactions.

Other aspects of the effects of metabolic poisons on the reactions of activation are not so readily interpreted; it is particularly odd, though not entirely without parallel, that germinal vesicle breakdown is initiated by exposure to iodoacetate or urethane (or, in a different manner, by application of Cu^{++} or *p*-chloromercuribenzoate), which substances are generally recognized as inhibitors or narcotics. The most evident interpretation of these results in the light of the related observations is that these inhibitory agents may prevent some alternative reactions of the activator substance or its precursors, so that the activator concentration is increased by the presence of the inhibitors. Obviously no specific characterization of the hypothetical reactions involved can be made, except that it seems likely that some enzyme concerned contains active sulfhydryl groups.

Some special mention should be made of the fact that there is not complete agreement in the results obtained with the various procedures employed. Some of these discrepancies are easily dismissed as quantitative differences in effects of the activators and inhibitors on the critical reaction rates. However, the distinct inhibition of citrate activation by *o*-nitrophenol is entirely out of line with all other relevant data; citrate in stimulatory concentrations also invariably failed to hasten sensitization of the eggs in trinitrophenol.

Beyond the considerations outlined, the present data do not permit identification of the hypothetical substances or their reactions; perhaps some clue is afforded in the apparent specificity of the *p*-nitrophenol grouping in the reversible formation of an inactive complex with the activator. The author is not prepared to interpret this finding; innovation in experimental approach is probably necessary

before a more coherent pattern will emerge from the diverse observations reported in this paper and in the earlier report. The interpretations offered seem the least involved and most comprehensive of the facts available at this time.

Summary

1. Inhibition of activation of *Nereis* eggs by trinitrophenol, with concurrent sensitization of the eggs to subsequent stimulation, appears to depend on the nitro-group in the para-position on the phenol.

2. The rate of the sensitization process is enhanced by anoxia, CO_2, inhibitors of the cytochrome system, or increased potassium ion concentration, but is insensitive to several other inhibitors, narcotics, stimulating agents, and to calcium ion deprivation.

3. The immediate initiation of activation by various chemical procedures requires the presence of the calcium ion, is assisted by the potassium ion, and slightly depressed by increasing magnesium ion concentration, but is not affected by anoxia or by various metabolic poisons.

4. Subsequent nuclear and cytoplasmic reorganization, ensuing some minutes after the initial disturbance, is reversibly inhibited by anoxia, inhibitors of the cytochrome system, or diethyl ether.

5. Urethane and iodoacetate activate the eggs; this activation is inhibited by trinitrophenol. Cupric ion and *p*-chloromercuribenzoate also activate the eggs, but only at nearly lytic concentrations, and the activation is not affected by trinitrophenol.

6. These data are partly interpreted in relation to the hypothesis of an activator metabolite produced within the egg.

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